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(54) Title: **ACYCLOVIR DERIVATIVES FOR TOPICAL USE**

(57) Abstract

Compositions for topical use in herpes virus infections comprising anti-herpes nucleoside analogue phosphate esters, such as acyclovir monophosphate, acyclovir diphosphate, and acyclovir triphosphate which show increased activity against native strains of herpes virus as well as against resistant strains, particularly thymidine kinase negative strains of virus. Anti-herpes nucleoside analogues phosphate esters include the phosphoramidates and phosphothiorates, as well as polyphosphates comprising C and S bridging atoms.

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ACYCLOVIR DERIVATIVES FOR TOPICAL USE

5 Acyclovir (ACV) is an antiviral nucleoside analogue of guanosine which contains an unusual incomplete (acyclic) sugar moiety. Nucleoside analogues interrupt the process of DNA replication in cells, and are for that reason useful as antiviral and antineoplastic agents. ACV is particularly effective in treating herpes simplex virus infections of types I and II. The antiherpes virus activity of ACV in cells occurs with low toxicity because ACV is selectively phosphorylated by HSV thymidine kinase, but not host cell thymidine kinase. As a consequence, only cells infected with HSV can form ACV monophosphate (ACV-MP). ACV-MP is then anabolically converted by cellular enzymes to ACV triphosphate, the active agent that interferes with 10 viral replication. (Fyfe, J., et al., J. Biol. Chem. 253:8721-8727 (1978); Furman, P., et al., J. Virol. 32:72-77 (1979)).

15 The anti-herpes virus activity of acyclovir has been demonstrated in inhibiting the replication of herpes simplex virus in tissue culture cells (O'Brien, W., et al., Antimicrob. Agents and Chemother. 34:1178-1182 (1990); it has also been demonstrated in clinical studies wherein patients infected with HSV were treated with orally administered ACV (Whitley, R., Immunobiol. and Prophylaxis of Human Herpesvirus Infections, C. Lopez et al. (eds) Plenum Press, NY 1990; and Straus, S., Sexually Transmitted Diseases 16(2):107-113 (1989)).

20 Acyclovir is the treatment of choice for mucosal and cutaneous HSV infections, although patients receiving topical acyclovir therapy experience some reductions of their symptoms, healing is slow and incomplete (Spruance, S., et al., J. Infect. Dis. 146:85-90 (1982); and Spruance, S., et al., Antimicrob. Agents Chemother. 25:553-555 (1984)).

25 Combination treatment using ACV together with interferon for herpes virus infected cultured cells (O'Brien, W., et al., Antimicrob. Agents and Chemother. 34(6):1178-1182 (1990) or using ACV together with A1110U, an HSV inactivator, as a topical therapy for herpetic keratitis in athymic mice (Lobe, D., et al., Antiviral Research 15:87-100 (1991) showed synergistic antitherpesvirus I activity over the use of ACV alone.

30 Acyclovir has been used with qualified success in a clinical trial to treat another viral disease, varicella (chickenpox) (Whitley, R., et al., Immunobiology and Prophylaxis of Human Herpesvirus Infections, C. Lopez (ed), Plenum Press, New York (1990) pp. 243-253. It has also been used experimentally but without success in treating other disorders in which a viral etiology was hypothesized, such as aplastic anemia (Gomez-Almaguer, D., et al. Amer. J. of Hematology 29:172-173 (1988) and duodenal ulcer (Rune, S.J., et al., Gut 31:151-152 (1990)).

35 Acyclovir phosphates have been shown to be efficacious against wild type or laboratory isolates of HSV-1 infected cultured cells in vitro, but have little or no efficacy against thymidine kinase defective mutants of HSV under the same conditions. (See data of Figures 1 and 2).

35 In immunosuppressed patients, such as those with HIV (AIDS) infections or transplant recipients who are taking immunosuppressive drugs to prevent transplant rejection, ACV has been given chronically to prevent

troublesome outbreaks of herpes. Such therapy provides a selective pressure which leads to mutations in HSV thymidine kinase (90% frequency) as well as DNA polymerase (10% frequency), which in turn results in ACV-resistant viral strains. There is no effective topical therapy for these acyclovir resistant herpes virus strains.

5 According to the invention, there are provided acyclovir phosphate esters and other antitherpes antiviral nucleoside analogue phosphate esters that are effective in the treatment of mucosal and cutaneous herpetic lesions due to herpes virus infections. These agents surprisingly show antiviral activity against lesions due to thymidine kinase defective herpes virus infections, even though they are relatively inactive against these mutant viruses in cultured cells. The invention also provides pharmaceutical formulations, 10 comprising an effective, antiviral concentration of an acyclovir derivative which can be acyclovir monophosphate, acyclovir diphosphate, acyclovir triphosphate, acyclovir monophosphate glycerol, acyclovir diphosphate glycerol, acyclovir monophosphate morpholidate, acyclovir diphosphate morpholidate, acyclovir monophosphate isopropylidene glycerol, acyclovir diphosphate isopropylidene glycerol, acyclovir phosphomethylenediphosphonate, or a mixture thereof, in a pharmaceutical carrier suitable for topical use.

15 Other antitherpes simplex nucleosides which rely on phosphorylation by viral thymidine kinase will also exhibit enhanced activity when applied to the skin of infected patients as their phosphate esters in a suitable topical formulation.

20 According to another aspect of the invention, there is provided a method for the topical treatment of a viral infection, comprising applying a formulation comprising any of the acyclovir phosphate derivatives of the invention, or a mixture thereof, to the mucosal or cutaneous lesions of a virus infected animal, including a human or other mammal. In a preferred embodiment of the method, the animal is infected with a herpes virus. In a particularly preferred embodiment, the animal is infected with a herpes virus strain that is resistant to acyclovir. The acyclovir resistant herpes virus strain can be a viral strain in which resistance to the antiviral agent is due to an alteration or defect in the thymidine kinase gene.

25 In accordance with another aspect of the present invention, at least one anti-herpes nucleoside analogue phosphate is used in the preparation of a medicament for the treatment of a mucosal or cutaneous viral infection. In a preferred embodiment, the nucleoside phosphate is a water soluble salt. In another preferred embodiment, the viral infection is herpes simplex virus, type 1 or type 2.

30 In another aspect of the present invention, the anti-herpes nucleoside analogue phosphate esters according to the invention are used together with a pharmaceutically acceptable carrier in the preparation of a medicament for the treatment of a mucosal or cutaneous viral infection. In a preferred respect, the pharmaceutically acceptable carrier is selected from the group consisting of an aqueous cream and polyethylene glycol.

35 Also provided are anti-herpes nucleoside phosphate esters such as acyclovir phosphoramidates and phosphothiorates and anti-herpes nucleoside analogue polyphosphate esters comprising sulfur and methylene bridging groups.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the comparative effect of acyclovir and acyclovir monophosphate on herpes simplex virus replication in WI38 fibroblasts.

5 Figure 2 illustrates the comparative effect of acyclovir and acyclovir monophosphate on the replication of HSV-DM.21 TK mutant in vitro.

Figure 3 illustrates the comparative effect of topical acyclovir and acyclovir phosphate esters on acyclovir-resistant HSV-1 infections of the TK-deficient type in HRS/J mice.

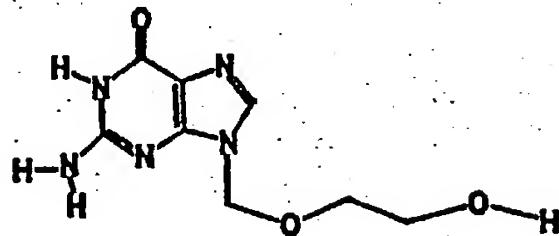
10 Figure 4 illustrates the comparative effect of topical acyclovir and acyclovir phosphate esters on acyclovir-resistant HSV-1 infections of the TK-altered type in HRS/J mice.

Figure 5 illustrates the comparative effect of topical acyclovir and acyclovir monophosphate on acyclovir-resistant HSV-1 infections of the wild type in HRS/J mice.

15 Figure 6 illustrates the comparative effect of topical acyclovir and acyclovir monophosphate on acyclovir-resistant HSV-1 infections of the TK-altered type in HRS/J mice.

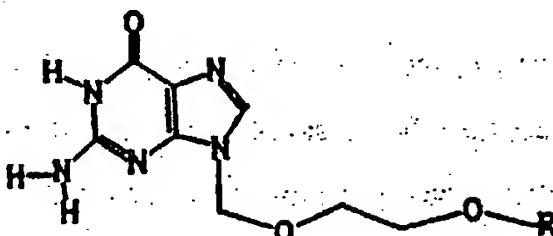
The present invention provides acyclovir phosphate derivatives that demonstrate excellent topical activity against herpes simplex virus (HSV) infections, particularly against ACV-resistant mutants of HSV.

20 Acyclovir is an analogue of the purine base, guanine, having a substituent group at the 9-position, and having an acyclic sugar group from which the common name is derived. The chemical name of acyclovir is 9-(2-hydroxyethoxymethyl)-guanine, which has the structure:



25

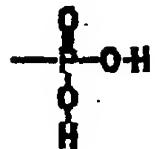
The acyclovir phosphate derivatives of the invention have a substituent, R, at the terminal O-locant of the acyclic sugar group, as follows:



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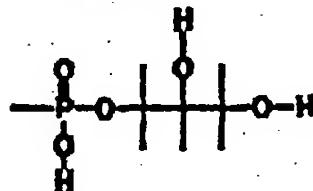
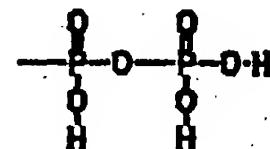
wherein the R substituents are as follows:

5

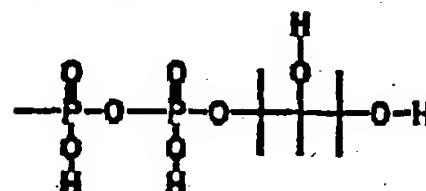


-monophosphate

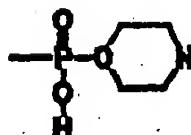
10

-monophosphate
glycerol

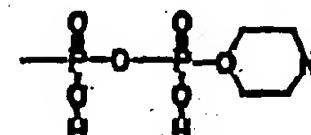
-diphosphate

-diphosphate
glycerol

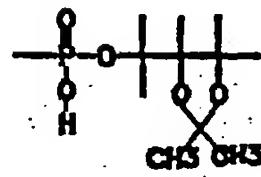
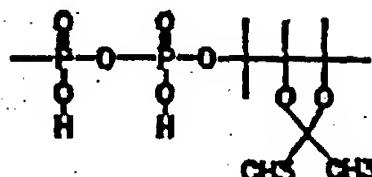
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20

-diphosphate
morpholidate

25

-monophosphate
isopropylidene
glycerol-diphosphate
isopropylidene
glycerol

30

Related triphosphate derivatives have corresponding structures comprising the additional phosphate group.

35

According to the invention, acyclovir monophosphate (ACV-MP), acyclovir diphosphate (ACV-DP), acyclovir triphosphate (ACV-TP), acyclovir monophosphate glycerol (ACV-MP-G), acyclovir diphosphate glycerol (ACV-DP-G), acyclovir monophosphate morpholidate (ACV-MP-morpholine), acyclovir diphosphate morpholidate (ACV-DP-morpholine), acyclovir diphosphate morpholidate (ACV-DP-morpholine), acyclovir phosphomethylenediphosphonate (ACV-PMDP), acyclovir monophosphate isopropylidene glycerol (ACV-MP-isoP-G), acyclovir diphosphate isopropylidene glycerol (ACV-DP-isoP-G), either alone or combined, are prepared in a suitable topical pharmaceutical formulation and applied to the cutaneous lesions of an HSV-infected individual. The compounds ACV-MP, ACV-DP, ACV-TP, ACV-DP-G, ACV-PMDP, the morpholine

derivative of acyclovir, and the acyclovir isopropylidene glycerol derivatives described, are non-lipid, water soluble phosphate esters, and are therefore preferably provided in an aqueous base topical formulation.

Surprisingly, we have discovered that the phosphate esters of acyclovir, and we expect that monophosphate and polyphosphate derivatives of other nucleosides will, exhibit enhanced topical anti-HSV activity. We have also demonstrated that salts of monophosphate, diphosphate and triphosphate and phosphomethylenediphosphonate derivatives of nucleoside analogues can be easily prepared, and that such salts exhibit enhanced solubility in aqueous media, i.e., cream, gels, or other aqueous dispersions. Moreover, such salts are soluble in polyethylene glycol media which provides a unique mucosal or cutaneous dispersion.

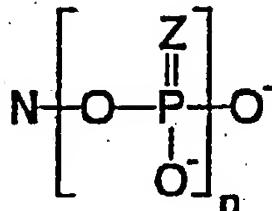
Other polyphosphate esters of nucleotide analogues that are useful in the methods of the invention include methylene- and thiolinked polyphosphate nucleoside analogues as well as mono- and polyphosphoamidate and mono- and polyphosphothiorate nucleoside analogues.

Similarly, monophosphates, diphosphates, and other phosphate esters of other antiherpes simplex nucleosides will exhibit enhanced topical activity as those above noted. The following herpes antiviral nucleosides exhibit enhanced activity as phosphate esters:

15 1-beta-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil;
 2'-fluorocarbocyclic-2'-deoxyguanosine;
 6'-fluorocarbocyclic-2'-deoxyguanosine;
 1-(beta-D-arabinofuranosyl)-5(E)-(2-iodovinyl)uracil;
 (1*r*-1*a*, 2*B*, 3*a*)-2-amino-9-(2,3-bis(hydroxymethyl)cyclobutyl)-6*H*-purin-6-one;
20 9*H*-purin-2-amine, 9-((2-(1-methylethoxy)-1-((1-methylethoxy)methyl)ethoxy)methyl)-(9CI);
 trifluorothymidine;
 9-((1,3-dihydroxy-2-propoxy)methyl)guanine;
 5-ethyl-2'-deoxyuridine;
 E-5-(2-bromovinyl)-2'-deoxyuridine;
25 5-(2-chloroethyl)-2'-deoxyuridine;
 1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-iodocytosine (FIAC);
 1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-iodouridine (FIAU);
 buciclovir;
 6-deoxyacyclovir;
30 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine;
 E-5-(2-iodovinyl)-2'-deoxyuridine;
 5-vinyl-1-beta-D-arabinofuranosyluracil (V-araU);
 1-beta-D-arabinofuranosylthymine (ara-T);
 2'-nor-2'-deoxyguanosine (2'NDG);
35 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine (penciclovir, BRL 3912);
 1-beta-D-arabinofuranosyladenine (ara-A; vidarabine)

The monophosphate, diphosphate and triphosphate esters have the general formula

5



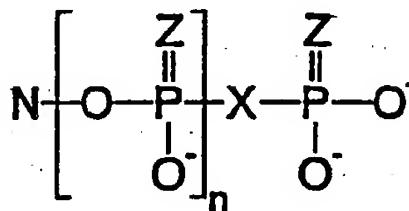
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wherein N is an anti-herpes simplex virus nucleoside analog;

Z is O, S, or NH; and n is 1 or 2; or alternatively have the following formula

15

20



25

wherein N is an anti-herpes simplex virus nucleoside analog;

Z is O, S or NH;

X is O, CH₂ or S; and

n is 1 or 2.

30

Accordingly, the phosphoesters can be phosphate, phosphothiorate, or phosphoramidate, and the diesters and triesters may have bridging atoms other than oxygen, for example, 2,3- μ -thiotriphosphate esters; or 2,3- μ -methylenediphosphonate.

35

Contrary to expectation, these nucleoside analog phosphates can pass through the cell membrane of HSV-infected skin cells and reduce the rate of viral replication by inhibiting the HSV DNA polymerase.

40

The mono-and diphosphate nucleosides of the invention are converted to their triphosphates by cellular anabolic phosphorylation(s) but the triphosphate analogs inhibit the HSV DNA polymerase directly without the need to inhibit cellular DNA polymerase. The invention also provides for pharmaceutical formulations of the nucleoside analog mono-, di- and triphosphates in concentrations that can be applied topically to effectively reduce the proliferation of HSV in infected skin cells. DNA chain-terminating dideoxynucleoside phosphates, when applied to the skin in a suitable topical formulation, will similarly reduce HSV replication. These include acyclovir, ganciclovir, penciclovir, BVarAU, dideoxycytidine, dideoxythymidine, dideoxyguanosine, dideoxyadenosine, dideoxyinosine, 3'-azidodideoxythymidine, dideoxyhydrodideoxythymidine (d4T) and other dideoxynucleoside analogs such as those described in copending US patent application SN 07/373,088.

5 Salts of these compounds can be easily prepared and such salts should exhibit enhanced solubility in aqueous media, i.e., cream, gels, or other aqueous dispersions. Typically, useful salts of these compounds include sodium, potassium, lithium, ammonium, or hydrogen salts. Any physiologically acceptable cation known to those skilled in the art may also be used. Moreover, such salts are usable and effective in polyethylene glycol creams and lotions which provide a favorable mucosal or cutaneous dispersion.

The various phosphate esters of these compounds may be prepared essentially as described below for acyclovir.

Synthesis of Acyclovir Phosphate Esters:

10 The present invention provides methods for the synthesis of acyclovir mono- and diphosphates, acyclovir monophosphate morpholidates, acyclovir mono- and diphosphate glycerols, and acyclovir mono- and diphosphate 1,2-isopropylidene glycerol.

15 Acyclovir monophosphate can be prepared from acyclovir according to the method of Yoshikawa, M., et al., Bull. Chem. Soc. Japan 42:3505-3508 (1969) as modified by the method of Toorchen, D. and Topal, M., Carcinogenesis 4:1591-1597 (1983), and exemplified in Example 1. Acyclovir diphosphate can be prepared, in the manner of other nucleoside analogues, by the method of Ott, D.G., et al., Anal. Biochem. 21:469-472 (1967), using either tributylammonium phosphate or tributylammonium pyrophosphate as the phosphate donor.

20 Methods for the preparation of acyclovir diphosphate glycerol are presented in Examples 2 through 4. In general, the nucleoside phosphate glycerols are prepared in a manner similar to that for the preparation of phosphatidyl nucleosides. In the approach described in Example 3, acyclovir phosphate is activated by the addition of a leaving group, for example, morpholine, according to Example 2, and condensed with glycerol-3-phosphate dicyclohexylammonium salt in the presence of N,N'-dicyclohexylcarbodiimide (DCC). Alternatively, as described in Example 4, glycerol phosphate, having the reactive hydroxyl groups protected by an isopropylidene moiety, is activated by addition of morpholide, and then condensed with acyclovir monophosphate under the conditions described for Example 2.

25 A number of acyclovir-diphosphate-diglycerides (ACV-DP-DG) containing various acyl chains have been prepared in the past, by the condensation of the appropriate diacylphosphatidic acid morpholidate (PA-Morpholidate) and acyclovir monophosphate (ACV-MP). A method by which the procedure can be carried out is described by Agranoff, B. and Suomi, W., Biochem. Prep. 10:47-51 (1963). Alternatively, the morpholidate of the nucleoside monophosphate is prepared and condensed with a phosphatidic acid as described in U.S. Patent Application Serial No. 07/706,873 entitled "Liponucleotide Synthesis," and by Hong, et al., British Patent Application No. 2,168,350.

30 The chemical methods above are generally disclosed in terms of their general application to the preparation of compounds of the invention. Occasionally, the reaction may not be applicable as described to each compound included within the disclosed scope. The compounds for which this occurs will be readily recognized by those skilled in the art. In all such cases, either the reactions can be successfully performed

by conventional modifications known to those skilled in the art, e.g. by appropriate protection of interfering groups, by changing to alternative conventional reagents, or by routine modification of reaction conditions. Alternatively, other reactions disclosed herein or otherwise conventional will be applicable to the preparation of the corresponding compounds of the invention. In all preparative methods, all starting materials are known or readily preparable from known starting materials. Unless otherwise indicated, all parts and percentages are by weight.

Synthesis of Nucleoside Monophosphates, Diphosphates and Triphosphates and Nucleoside Phosphate Analogues:

The methods for synthesizing nucleoside monophosphates by reacting the nucleoside with phosphorus oxychloride are described in copending U.S. patent applications SN 07/373,088 and SN 08/060,258 and as previously described (Yoshikawa et al., Bull. Chem. Soc. Japan 42:3505-3508, 1969; Toorchen, D. and Topal, M., Carcinogenesis 4:1591-1597, 1983). Nucleoside diphosphates are prepared by the method of Ott, D.G. et al (Anal. Biochem. 21:469-472, 1987).

Nucleoside triphosphates are prepared by the method of Seela and Röling (Nuc. Acids Res. 20:55-61, 1992), or from the nucleoside monophosphates by the method of Moffat and Khorana (J. Am. Chem. Soc. 83:663, 1991), or by the method of Hoard and Ott (J. Am. Chem. Soc. 87:1785-1788, 1963). The examples below present the details of some syntheses useful for preparing phosphate esters of nucleosides and nucleoside analogs.

Other nucleoside phosphate analogues including nucleoside phosphorothioates, nucleoside phosphoramidates, nucleoside phosphonates and nucleoside phosphorofluoridates can be synthesized using methods well known to those skilled in the art and summarized, for example, by D.W. Hutchinson (The synthesis, reactions and properties of nucleoside mono-, di-, tri-, and tetraphosphates and nucleotides with changes in the phosphoryl residue. In Chemistry of Nucleotides and Nucleosides, L. Townsend, ed., 1991 at pp 81-146 and references therein). The common syntheses are summarized as follows.

(1) Nucleoside Phosphorothioates are analogues of nucleotides in which one or more of the phosphoryl oxygen atoms have been replaced by sulfur. Early methods of synthesis reacted a protected nucleoside and tris(1-imidazolyl)phosphane sulfur whereas more recent syntheses replace the latter reagent with thiophosphoryl-chloride (PSCl_3). A nucleoside phosphoramidate can be converted into a phosphorothioate by treatment with sodium hydroxide and carbon disulfide. Nucleoside 5'-phosphorothioates can result from direct sulfurization of nucleoside 5'-phosphites. Purine nucleoside 2'(3')-phosphorothioates can be synthesized by reacting their 2',3'-O-di-n-butylstannylene derivatives with thiophosphoryl chloride followed by alkaline hydrolysis.

(2) Nucleoside Phosphoramidates are analogues in which one or more phosphoryl oxygen atoms have been replaced by nitrogen creating a P-N bond which is considerably more labile than the P-S bond of nucleoside phosphorothioates even under mildly acidic conditions. Syntheses of these compounds include the

phosphorylation of aminonucleosides and the treatment of nucleoside azides with triesters of phosphorous acid. Lipophilic nucleoside phosphoramidates may be particularly useful anti-HSV compounds because of their ability to be more readily taken up by cells where they are hydrolyzed into biologically active compounds.

(3) Nucleoside Phosphonates are compounds in which a phosphoryl oxygen is replaced by carbon creating a stable P-C bond and having decreased acidity of the P-OH groups when the phosphorus atom is substituted with an electron-donating alkyl group in place of the oxygen. Nucleoside phosphonates are easily prepared from nucleoside halides by those skilled in the art using either the Arbusov or the Michaelis-Becker reactions. Nucleoside 5'-phosphonates can be synthesized from 2',3'-protected 5'-iodo-5'-deoxynucleosides using methods well known to those skilled in the art. Isosteric nucleoside 5'-phosphonates, in which the 5'-oxygen is replaced with a methylene group, are synthesized by coupling a suitably-protected nucleoside 5'-aldehyde with diphenyl triphenylphosphoranylidene(methyl)phosphonate to give a α,β -unsaturated phosphonate diester that is then reduced and deprotected at the phosphoryl residue to give the phosphonate. Isosteric nucleoside 3'-phosphonates are synthesized starting from the phosphorylated ribose-1 chloride which is coupled with the heavy metal salt of a purine or pyrimidine. Phosphonates are generally less polar than their phosphate counterparts and therefore are useful as anti-HSV agents because they are more readily taken up by cells when applied topically.

(4) Nucleoside Phosphorofluoridates are analogues of mononucleotides. Treatment of nucleoside 5'-phosphates with 2,4-dinitrofluorobenzene produces the nucleoside 5'-phosphorofluoridates via the 2,4-dinitrophenylester of the nucleotide.

(5) Other Nucleoside Polyphosphate Analogues include those in which atoms other than oxygen have been substituted between the α,β -phosphorus atoms in di- and tri-phosphates of nucleosides or between the β,γ -phosphorus atoms in nucleoside triphosphates (including those listed in Table III at page 119 of D.W. Hutchinson, In Chemistry of Nucleotides and Nucleosides, L. Townsend, ed., 1991). Usually, the α,β -analogues are prepared by condensing a 2',3'-O-protected nucleoside with the pyrophosphate analogue with the aid of DCC or by nucleophilic displacement reactions involving the displacement of a toluene-sulfonyl (tosyl) residue from the 5'-position of the sugar residue of the tosyl nucleoside by methylene bisphosphonate ion.

The acyclovir derivatives of the invention, comprising ACV-MP, ACV-DP, ACV-TP, ACV-MP-glycerol, ACV-DP-glycerol, ACV-MP-isopropylidene glycerol, ACV-DP-isopropylidene glycerol and ACV-P-methylenediphosphonate were found to have particular efficacy in topically treating the herpetic lesions of acyclovir-resistant HSV-1 infections. Example 7 demonstrates that infection of cultured cells with wild type isolates and laboratory strains of HSV can be treated with equal success using acyclovir, acyclovir monophosphate (Example 7; Figure 1). For these viral infections in WI38 fibroblasts, both acyclovir and acyclovir monophosphate have the same IC₅₀ of about 1 or 2 μ M concentration. However, when the same cultured cell system is infected with an acyclovir-resistant strain of virus, HSV-DM-21, lacking the thymidine

kinase necessary to convert acyclovir to acyclovir monophosphate, acyclovir and acyclovir monophosphate are ineffective in reducing the number of viral plaques (Example 7; Figure 2).

The efficacy of acyclovir phosphate esters with respect to acyclovir-resistant cutaneous HSV-1 infections is surprising in view of the cultured cell *in vitro* data above. Acyclovir phosphate esters applied in an aqueous cream to the herpetic lesions of mice infected with acyclovir-resistant HSV-1 were substantially more effective than native acyclovir in reducing the number of such lesions (Example 9; Figures 3 and 4).

Accordingly, in view of these results, it is believed that *in vitro* incorporation of acyclovir, and acyclovir phosphates, proceed through a different mode of operation than *in vivo* as a topically applied lotion.

10

Topical Formulations of Nucleoside Analogue Phosphates:

The nucleoside analogue derivatives of the invention as previously described can be prepared for topical use by incorporation into a variety of formulations known to those in the art as useful and convenient for dermatological use. The nucleoside analogue derivatives are water soluble, and accordingly an aqueous solution, water-in-oil emulsion, or an aqueous cream are highly preferred formulations. Water solubility of the acyclovir and other nucleoside monophosphates can be enhanced through the preparation of salts, such as sodium, potassium, ammonium, or hydrogen. In a particularly preferred formulation, the active ingredient is prepared in a polyethylene glycol (PEG) vehicle. Alternatively, the active ingredients can be topically applied in a dry powder formulation, using an insoluble powder, such as starch or talc as a diluent or carrier.

The vehicle is an important component of some topical formulations, because it can be selected to enhance penetration, to prolong the duration of activity, or to meet requirement of the site of application. For example, a formulation for application to the callous parts of the body, such as the palms of the hand or bottoms of the feet, can include a penetration enhancing agent such as dimethylsulfoxide propylene glycol or azone™; a powdery formulation can be selected for application to the intertriginous zones such as the crotch, inner elbow or between the fingers or toes. The formulation can also be made up to contain various organic polymers or other compositions known to those of skill in the art to give sustained release of the active antiviral acyclovir derivatives.

A multitude of appropriate topical formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences, 15th Edition, 1975. Mack Publishing Company, Easton, Pennsylvania 18042. (Chapter 87: Blaug, Seymour). These formulations include for example, powders, pastes, ointments, jelly, waxes, oils, lipids, anhydrous absorption bases, oil-in-water or water-in-oil emulsions, emulsions carbowax (polyethylene glycols of a variety of molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax.

The concentration of active ingredient in the topical formulations of the invention can be from about 0.01 gm% to 100 gm%; preferably from about 0.1 gm% to 50 gm%; most preferably from about 1 gm% to about 15 gm%. The formulations can further comprise effective concentrations of other agents which help to promote penetration of the skin and healing, as described in the above-referenced formulary and are

well known to those of ordinary skill in the art.

Efficacy of topical formulations containing the active phosphate esters of the invention can be evaluated using conventional testing procedures, known to those of skill in the art. For example, a particularly expeditious procedure is the murine "orofacial model," as described by Ellis, M., et al, 5 *Antimicrobial Agents and Chemotherapy* 33:304-310 (1989). In this test system, the pathogenesis of HSV in mice scarified and inoculated on the snout has been shown to be a reasonable model of the disease cycle of cutaneous HSV infection in the immunocompromised host.

The formulations can be applied to the herpetic lesions of the affected skin repeatedly; for example once, twice, or several times a day, and the treatment can be extended for several days until healing is 10 achieved. The risk of incidence of toxicity and irritation is minimal.

EXAMPLE 1

Phosphorylation Of Acyclovir Nucleoside Derivatives

Acyclovir nucleoside derivatives were prepared through the following methods. Unprotected acyclovir was reacted with POCl_3 in trimethyl phosphate ($(\text{CH}_3\text{O})_3\text{PO}$) essentially as described by Yoshikawa et al. 15 *Tetrahedron Letters* 50:5065-5068 (1967); and Yoshikawa, M., Kato et al. *Bull Chem. Soc. Japan* 42:3205-3208 (1967). To a cooled solution (0°C) of 2 M POCl_3 in 300-400 mL trimethyl phosphate, acyclovir (1 M) was added dropwise with stirring, the reaction temperature being held constant between 0° and 5°C . The progress of the reaction was monitored by means of HPLC using a Mono Q HR 5/5 anion 20 exchange column (Pharmacia, Uppsala, Sweden). Typically, 5 μL of the reaction mixture was neutralized with aqueous sodium hydroxide (final pH 7) and injected on the column.

Elution was performed as follows: washing with water, elution with 0.1 M ammonium carbonate, NH_4HCO_3 , which elutes the acyclovir monophosphate, followed by a linear gradient of 0.1-0.6 M NH_4HCO_3 which elutes some higher phosphorylated products. The reaction was mostly completed within 45 to 75 minutes as determined by this method, and the reaction product was hydrolyzed and neutralized with 25 2 volumes of aqueous sodium hydroxide to final pH of 7.

Purification of the product compound was conducted as described above for the analysis of the reaction mixture. By this method, 0.8 moles of acyclovir monophosphate were prepared and purified with a Q Sepharose fast flow column using the same elution conditions.

Yields varied between 80% and 96% after repeated lyophilization from water. 30 TLC analysis (Silica 60/F254 Plates, Merck) showed a single U.V. and Pi positive spot, using the developing system 1-propanol/25% $\text{NH}_3/\text{H}_2\text{O}$ (20:20:3 by volume)

EXAMPLE 2

Preparation Of Acyclovir-monophosphomorpholidate

Acyclovir-monophosphate (5 mmol) and morpholine (20 mmol) were suspended in t-butanol (50 mL) and heated under gentle reflux while *N,N'*-dicyclohexylcarbodiimide (DCC, 20 mmol) dissolved in t-butanol (50 mmol) was added dropwise over a period of 1 hour. The mixture was stirred under reflux for 12 to 36 hours.

and evaporated to dryness. The residue was triturated with ether and washed by decantation with the same solvent. The product was purified by recrystallization from methanol-ether mixtures.

EXAMPLE 3

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Preparation Of *sn*-Glycero-3-diphospho-acyclovir From Acyclovir-monophosphomorpholidate

Acyclovir monophosphomorpholidate (2 mmol), prepared as described in Example 2, was dissolved in dry pyridine (20 mL) and evaporated to dryness under vacuum. The process of dissolving the residue in pyridine and evaporation was repeated three additional times to remove traces of water from the compound. Glycerol-3-phosphate di-monocyclohexylammonium salt (3 mmol) was added to the dried residue and the mixture was dissolved in 20 mL of pyridine and stirred under inert atmosphere at 60° C for 12-36 hours. The solvent was evaporated under vacuum and the residue was titrated with ether and the resulting solid was purified by ion exchange chromatography over DEAE sephadex column (2.5 cm x 30 cm) using a linear gradient of ammonium bicarbonate (10 mmol to 300 mmol, 500 mL each). Fractions containing pure product identified by using TLC and analytical HPLC) were pooled and lyophilized to furnish the title compound.

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EXAMPLE 4

Preparation Of *sn*-Glycero-3-diphospho-acyclovir From 1,2-isopropylidene-*sn*-glycero-3- monophosphomorpholidate

A. Preparation Of 1,2-Isopropylidene-*sn*-glycero-3-phosphate

20 Phosphorous oxychloride (25 mmol) was added dropwise over a period of 30 minutes to a mixture of 1,2-isopropylidene-glycerol (20 mmol) (Sigma, St. Louis, MO.) and triethylamine (100 mmol) that was cooled to 0°C. After stirring the mixture at 0°C for 10 to 90 minutes. Water (1 mL) was added to stop the reaction. The mixture was then dissolved chloroform (500 mL) and washed with water (3 x 100 mL). The water wash solutions were combined and back extracted with chloroform (50 mL) and lyophilized. The product was used immediately for subsequent reactions without any additional purification.

B. Preparation Of 1,2-isopropylidene-*sn*-glycero-3-monophosphomorpholidate

1,2-isopropylidene-*sn*-glycero-3-phosphate, prepared as described in (A), was condensed with morpholine to prepare 1,2-isopropylidene-*sn*-glycero-3-monophosphomorpholidate, according to the procedure described for the preparation of acyclovir phosphomorpholidate in Example 2.

30 Reaction of the intermediate compound, 1,2- isopropylidene-*sn*-glycero-3-monophosphomorpholidate, with acyclovir monophosphate under the conditions described in Example 2, yielded 1,2-isopropylidene-*sn*-glycero-3-monophosphomorpholidate.

1,2-isopropylidene-*sn*-glycero-3-diphospho-acyclovir (1mmol) was dissolved in 50 to 80% aqueous acetic acid and stirred at room temperature for a period of 4 to 12 hours and the crude glycero-3-diphospho-acyclovir product was purified as described above.

EXAMPLE 5

Preparation of *sn*-glycero-3-phospho-acyclovir

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1,2-isopropylidene-*sn*-glycero-3-phosphate, 1 mM (prepared as in Example 4B), and acyclovir (1 mM),

-13-

were suspended in dry pyridine (10 mL) and DCC (4 mmol). Dissolved pyridine (4 mL) was added and the mixture stirred at 25°C to 60°C for 12 to 72 hours. The solvent was evaporated and the residue was titrated with ether. The crude product was purified by ion exchange chromatography as described in Example 3. The isopropylidene-protecting group was then removed from the product by treating with aqueous acetic acid to furnish the title compound.

5 Alternatively, the title compound was also prepared by using 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) as the condensing agent.

EXAMPLE 6

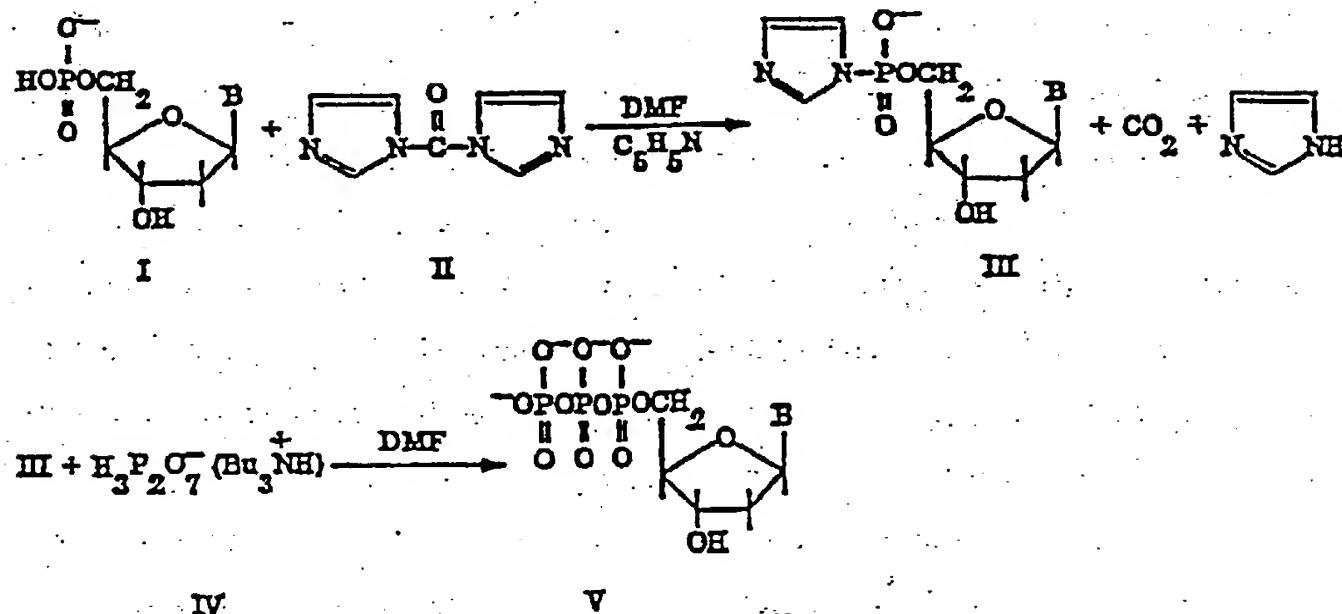
Preparation Of Acyclovir Phosphate Ester Mixture
 10 By The Alkaline Hydrolysis Of
Acyclovir Diphosphate Digalmitoylglycerol

Acyclovir-diphosphate-digalmitoylglycerol (1 mmol) was dissolved in chloroform, to which methanolic sodium hydroxide (2.1 mmol) was added. The reaction was carried for 20 to 90 minutes and the progress was monitored by TLC. Upon completion of the reaction, Dowex-50 X-2 (H⁺) was added to the reaction mixture to adjust the pH to 7. The resin was separated by filtration and the filtrate was lyophilized and the crude product was purified as described in Example 1.

EXAMPLE 7

Preparation of Nucleoside Triphosphates From Mononucleosides

20 Preparations of 5' triphosphates of deoxyribonucleotides, dideoxyribonucleotides and analogs involves a series of reactions as outlined immediately below.



30 Nucleotide analogue monophosphate synthesis is described in the co-pending U.S. patent application Serial No. 08/060,258, filed May 12, 1993, and in Example 1. Further methods are as follows:

A nucleotide (I) and excess 1,1'-carbonyldimidazole (II) are reacted for about 1 hour at room temperature to form an imidazolide (III). Unreacted 1,1'-carbonyldimidazole is decomposed with methanol before an excess of inorganic pyrophosphate (IV) is added. This eliminates the formation of inorganic polyphosphates which would have to be subsequently removed from the reaction materials. Phosphorylation is allowed to proceed to completion at about 24 hours after addition of the inorganic pyrophosphate (IV) and then the nucleoside triphosphate product (V) is purified by anion-exchange chromatography on DEAE-cellulose followed by conversion of the product to a salt such as a sodium salt. Because the nucleotide (I) and the imidazolide (III) can react together to form a symmetrical pyrophosphate by-product, the anion-exchange chromatography on DEAE-cellulose is carried out at a lower pH where the desired product (V) has less charge than the undesirable by-product, thus allowing separation of the two compounds.

One reagent used in the synthesis is tributylammonium pyrophosphate which is made by the following procedure. To an aqueous solution of pyridinium pyrophosphate, obtained by passing a solution of tetrasodium pyrophosphate decahydrate (446 mg, 1 mmole) through a column of Dowex 50W-X4™ (pyridinium) resin (17 mL) is added tributylamine (0.24 mL, 1 mmole). The solution is concentrated under vacuum and the residue is then dried by consecutive addition and evaporation of anhydrous pyridine followed by addition and evaporation of two 10-mL portions of N,N-dimethylformamide (DMF).

The synthesis of nucleoside triphosphates is accomplished by the following method. To a solution or suspension of the mono-nucleotide (0.1 mmole) as the anhydrous tributylammonium salt, in 1 mL of DMF, is added 1,1'-carbonyldimidazole (80 mg, 0.5 mmole) in 1 mL DMF. The combination is mixed for 30 minutes and then held in a desiccator at room temperature for 4-12 hr before it is treated with 33 μ L (0.8 mmole) of methanol and allowed to react for 30 min at RT. Tributylammonium pyrophosphate (0.5 mmole) in 5 mL DMF is added and vigorously mixed and then the mixture is held in a desiccator at RT for about 24 hr to allow imidazolium pyrophosphate to precipitate. The precipitate is removed and washed with four 1 mL portions of DMF by centrifugation and resuspension in the DMF resulting in about 80-100% purity. The supernatant is treated with an equal volume of methanol and evaporated to dryness under vacuum. The residue is chromatographed on a 2 X 20 cm column of DEAE-cellulose with a linear gradient of triethylammonium bicarbonate (a 3 L gradient of about 0 to 0.4M at pH from 5 to 7.5 and fractions are collected and assayed spectrophotometrically to identify fractions containing nucleoside triphosphates. The appropriate fractions are evaporated under vacuum and the triethylammonium nucleoside triphosphate is dissolved in methanol to a concentration of about 0.05 M and five volumes of an acetone solution of sodium perchlorate (15 equiv) is added to form a precipitate of the sodium salt of the nucleoside triphosphate. It will be understood by those skilled in the art that other salts of the nucleoside triphosphate could be made by the appropriate precipitation reactions. The precipitated salt is collected by centrifugation, washed with four 1 mL portions of acetone and dried under vacuum over phosphorus pentoxide.

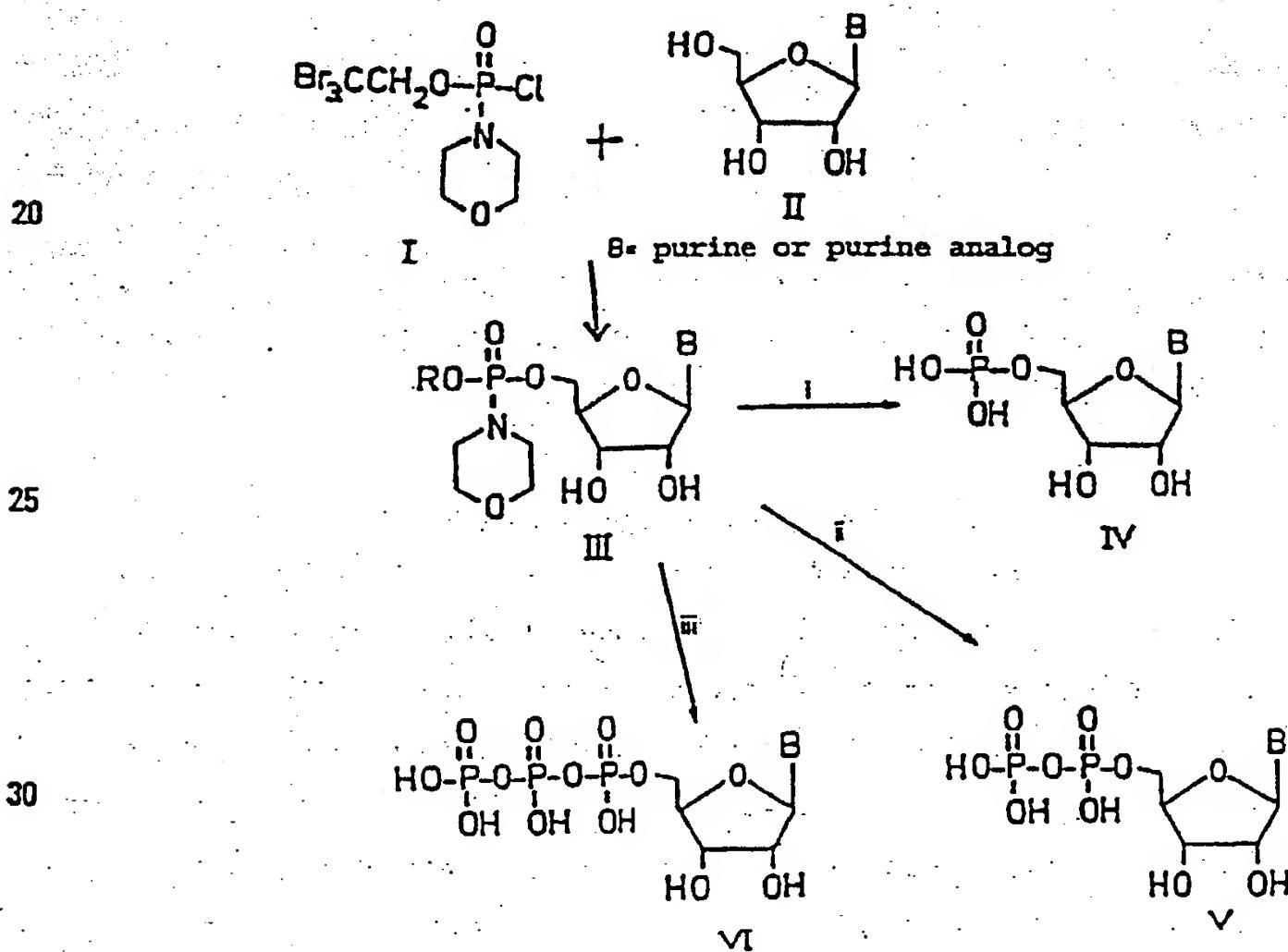
Additional procedures are available for synthesis of nucleoside triphosphates including the one presented in the next example.

EXAMPLE 8

Synthesis of Nucleoside Mono-, Di- and Tri-phosphates Using 2,2,2-Tribromoethyl Phosphoromorpholinochloridate

Using the method essentially of van Boom, et al. (Tetrahedron Lett. 32:2779-2782, 1975), mono-, di- and triphosphates of ribonucleosides and their derivatives are prepared from a single intermediate. In general, the reactions include reacting a monofunctional reagent (2,2,2-tribromoethyl phosphoromorpholinochloride) with a ribonucleoside (or its derivative) to make phosphotriester derivatives with a 2,2,2-tribromoethyl protecting group attached to the ribonucleoside (i.e., to produce a ribonucleoside 5'-phosphomorpholides or ribonucleoside 5'-phosphomorpholide derivatives). The protecting group is removed by a Cu/Zn coupling reaction with acidic deblocking and neutralization to produce the mono-, di-, and triphosphates depending on the acid used in the deblocking step and the ammonium salt used in the neutralizing step. That is, to obtain the monophosphate ribonucleoside, HCl and ammonia are used; to obtain the diphosphate ribonucleoside, the mono(*tri-n*-butylammonium) salt of phosphoric acid is used; to obtain the triphosphate ribonucleoside, bis(*tri-n*-butylammonium) pyrophosphate is used.

15 The general reactions are diagrammed as follows:



The monofunctional reagent (I) 2,2,2-tribromoethyl phosphoromorpholinochloride is prepared by reacting 2,2,2-tribromoethyl phosphorodichloride and morpholine in anhydrous ether, from which the reaction product is removed and recrystallized using cyclohexane/n-pentane using methods well known in the art. The crystalline 2,2,2-tribromoethyl phosphoromorpholinochloride has a mp of 79°C.

The monofunctional reagent (2 mmole) is mixed with 1 mmole of the nucleoside or its derivatives in anhydrous pyridine at 20°C for 48 hr; then the reaction mixture is fractionated chromatographically (B.J. Hunt & W. Rigby, *Chem. & Ind.* 1868, 1967) to yield colorless solids of nucleotides (III). Treatment of the nucleotides with Cu/Zn couple in anhydrous DMF for 10 min at 20°C followed by filtration to remove excess Cu/Zn gives the nucleoside phosphoromorphosidates.

5 The nucleoside phosphoromorphosidates are then treated with different acids and ammonia sources to yield either mono-, di-, or triphosphates. For monophosphate, the phosphoromorphosidate is treated with 0.01 N HCl, pH 2 for 2 hr at 20°C and then neutralized with aqueous ammonia (pH 9) and purified over a column of Sephadex G-25™.

10 Similarly the nucleoside 5'-triphosphate is obtained by reacting the phosphoromorphosidate (0.1 mmole) in 2 mL of anhydrous DMF with 0.5 mmole of bis(*tri-n*-butylammonium) pyrophosphate in 2 mL of anhydrous DMF at 20°C for 3 hr under conditions that exclude moisture. The reaction product is concentrated under vacuum, treated with Dowex 50™ (ammonium form), and purified on a 2 x 25 cm column of DEAE cellulose using a 3 L linear gradient of 0.0 to 0.3M Et₃NH₂CO₃ solution.

15 The nucleoside 5'-diphosphate is obtained by reacting the phosphoromorphosidate (0.1 mmole) with 0.6 mmole of mono(*tri-n*-butylammonium)phosphate in 4 mL of anhydrous pyridine at 20°C for 3 hr under conditions that exclude moisture and the reaction product is similarly concentrated and purified. Alternatively, the phosphotriester derivatives (III) can be converted directly into the corresponding nucleoside diphosphates by treatment with Zn dust in pyridine solution containing mono(*tri-n*-butylammonium)phosphate. That is, 1

20 mmole of reagent III is added to a stirred solution of 15 mL anhydrous pyridine containing 0.1 g of finely divided Zn and 12 mmole mono(*tri-n*-butylammonium)phosphate under conditions that exclude moisture for about 48 hr at 20°C. Then the reaction mixture is centrifuged to pellet the Zn and the supernatant is co-evaporated three times with 15 mL of water at each step before purification on DEAE cellulose.

EXAMPLE 9

Synthesis of Acyclovir Triphosphates

(1) *Acyclovir-5'-triphosphate, triethylammonium salt, synthesis.* Acyclovir (25 mg, 0.1 mmol) is dissolved in trimethyl phosphate (250 μ L, 1.07 mmol) and POCl₃ (18.5 μ L, 0.2 mmol) are added. The mixture is stirred for 1.5 h at 0°C and then a mixture of 0.5 M bis(*tri-n*-butylammonium) pyrophosphate in 1 mL of anhydrous DMF and 1 mL of *tri-n*-butylamine are added with vigorous stirring for 1 min.; the solution is neutralized with 1 M aqueous Et₃NH₂CO₃ solution, pH 7, and evaporated to dryness under vacuum. The residue is purified on a 2.6 x 30 cm column of DEAE-sephadex using a linear gradient of Et₃NH₂CO₃, pH 7 (11 H₂O/11 0.7 M TBK) solution to yield a colorless solid with a UV (H₂O) λ_{max} of 258 nm.

EXAMPLE 10

Synthesis of Acyclovir-phosphomethylenediphosphonate

35 The synthesis is essentially as described in Method 1 of Myers et al. (*J. Am. Chem Soc.* 85:

3292-3295, 1963). A nucleoside 5'-phosphoramidate is reacted with methylenediphosphonic acid to produce the phosphonic acid analogs of the nucleoside polyphosphate. Alternatively, using Method 2 of Myers et al. (id.), a nucleoside monophosphate is reacted with methylenediphosphonic acid using dicyclohexylcarbodiimide (DCC) as the condensing agent.

5 According to Method 1, methylenediphosphonic acid is obtained by hydrolysis in concentrated HCl of its tetraethyl ester which is prepared by the reaction of methylene iodide with excess triethyl phosphite. 1,3-Dicyclohexylguanidinium acyclovir 5'-phosphoramidate (3.6 mmole) and methylenediphosphonic acid (10.8 mmole) are treated with 54 mL of freshly distilled *o*-chlorophenol; the mixture is cooled on ice and 36 mL of dry pyridine is added. This resulting solution is allowed to stand at RT with occasional shaking for 48 hr when 300 mL of water is added while cooling in ice. The solution is extracted six times with ether (850 mL total). The aqueous solution is adjusted to pH 2 with 1 N HCl and then treated with 30 g of acid-washed charcoal (Norit A) and stirred for 30 min; then the charcoal is collected by filtration and washed exhaustively with water (5 L total vol). The acyclovir derivative is eluted with 50% aqueous ethanol-5% concentrated ammonium hydroxide (3 L total) and the eluate is concentrated to a 400 mL volume by evaporation at 35°C. The concentrated eluate is applied to a 2.7 cm x 31 cm column of Dowex-2™ (chloride; 8% cross linking) and eluted with a linear gradient made from mixing 2 L of 0.003 N HCl (in the mixing vessel) and 2 L of 0.003 N HCl plus 0.45 N LiCl (in the reservoir); 10 mL fractions are collected and fractions containing the acyclovir methylenediphosphonate are identified using paper chromatography or ultraviolet absorbance using methods well known in the art. The acyclovir methylenediphosphonate containing fraction is neutralized with 1 N LiOH and concentrated by evaporation at 30°C; the concentrated solution is treated with 250 mL of acetone-10% methanol to precipitate a solid which is separated by centrifugation and washed with the acetone-10% methanol mixture until no chloride is detected in the washes. The Li-salt of acyclovir methylenediphosphonate can be further purified by dissolving the salt in 100 mL of water adjusted to pH 8 with LiOH and chromatographing the solution through a Dowex-2™ column as described above using a gradient made from 1.5 L of 0.003 N HCl in the mixing chamber and 1.5 L of 0.003 N HCl plus 0.45 N LiCl in the reservoir and treating the eluate as described above followed by dissolving the precipitate in 6 mL water and precipitating it with 40 mL of methanol. The final precipitate is dissolved in 15 mL of water and lyophilized to produce a powder of tetra lithium acyclovir methylenediphosphonate.

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Using Method 2, methylenediphosphonic acid (11.4 mmole) and acyclovir monophosphate (2.6 mmol) are dissolved in pyridine (30 mL) and water (4 mL) to produce a two-phase mixture to which DCC is added at RT with vigorous stirring in three aliquots (29 mmole at the start of the reaction; 48 mmole after 4 hr; and 19 mmole after 12 hr). After 24 hr, the reaction is completed and precipitated dicyclohexylurea is filtered off and washed with water. The filtrate and washings are adjusted to a total volume of 150 mL with water and extracted five times with ether (300 mL total). The solution is adjusted to pH 8 and chromatographed on a 2.5 cm x 17.5 cm column of Dowex-1™ (formate; 2% cross linking) column; the column is washed with 1.5 L of water to remove pyridine. Elution from the column is carried out using a

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gradient created by adding successively to a mixing chamber containing 500 mL water the following solutions: 4 N formic acid (500 mL), 4 N formic acid plus 0.1 M ammonium formate (500 mL), and 4 N formic acid plus 0.2 M ammonium formate (1500 mL) and collecting 15 mL fractions. The fractions containing 2CdATMDP (approximately in tubes 115-134) are identified using ultraviolet absorption using methods well known in the art. The combined fractions containing 2CdATMDP are lyophilized to a volume of about 200 mL and then treated with 7 g of acid-washed charcoal (Norit A) and stirred for 15 min; then the charcoal is collected by filtration and washed with water (800 mL total). The product is eluted with 50% aqueous ethanol-5% concentrated ammonium hydroxide (600 mL total) and the eluate is concentrated to a 200 mL volume by evaporation at 20°C, filtered to remove trace amounts of charcoal and lyophilized to powder. The powder is dissolved in 4 mL of water and the solution is treated with excess 1 M barium acetate; the resulting precipitate is collected by centrifugation, washed with water and dissolved in 0.1 N HBr at 0°C. The solution is adjusted to pH 6.5 with 1 N NaOH and the resulting precipitate is collected by centrifugation, washed with successively with 2 x 2 mL each of water, ethanol and ether. The sample is dried at RT over P₂O₅ for 12 hr to yield dibarium 2CdATMDP hydrate. Other nucleoside analog phosphomethylenediphosphonates of the invention are prepared similarly.

EXAMPLE 11

Absence Of Antiviral Effect Of Acyclovir Monophosphate In Acyclovir-Resistant TK Mutant Strains Of HSV (DM.21)

Separate cultures of WI-38 fibroblast cells infected with either wild type strains of herpes simplex virus (HSV) or a mutant strain of HSV (DM.21) were individually treated with acyclovir, or acyclovir monophosphate. The DM.21 mutant lacks the thymidine kinase enzyme which usually converts ACV to ACV-MP, and is therefore resistant to acyclovir. The results for HSV-1 are shown in Figure 1, and those for HSV-DM.21 are shown in Figure 2. An IC₅₀ is that concentration of antiviral agent which inhibits viral plaque formation 50%.

In wild type isolates and laboratory strains of herpes simplex virus (HSV-1), acyclovir and acyclovir monophosphate have IC₅₀s of 0.1 μ M (Figure 1). In contrast, both acyclovir and acyclovir monophosphate have IC₅₀s in excess of 100 μ M against mutant HSV strains in this assay (Figure 2).

Based on these results in vitro, one would not expect acyclovir monophosphate to exhibit significant activity when administered topically to animals infected with a thymidine kinase defective or other mutant strain of HSV.

EXAMPLE 12

Antiviral Effect Of Acyclovir Phosphate Esters In Mice Infected With Acyclovir-Resistant Strains Of HSV

Mice of the HRS/J type were infected cutaneously using the snout scarification method as described by Ellis, M. et al, Antimicrobial Agents and Chemotherapy 33(3):304-310 (1989). Briefly, groups of 10 mice, under light ether anesthesia, were inoculated on the snout by scarification with a 25-gauge needle, followed by 10 seconds of rubbing with a cotton-tipped applicator soaked in diluted virus. The virus used for infection

was a TK-deficient strain, referred to in Ellis, M. et al. (TK^D). Three hours post-infection, the animals were treated topically, 3 times daily, for 4 days, with formulations of acyclovir or acyclovir phosphates, in a aqueous cream (AC), according to the Ellis reference cited above.

5 The results are presented in Figure 3. A formulation comprising 5 gm% acyclovir was active. In contrast, a formulation comprising 5 gm% of a mixture of 80% acyclovir monophosphate together with 20% other acyclovir phosphate esters (acyclovir diphosphate and acyclovir diphosphate glycerol) showed superior activity, with only a few mice developing herpetic lesions. All lesions were healed by day 8 in all groups.

10 The above procedure was repeated, with treatment continuing for 5 days, using the TK-altered HSV-1 virus (TK^A, Ellis, above), a more virulent strain. Unlike the TK^D virus, TK^A is fatal in untreated mice. Treatment with 5 gm percent acyclovir reduced lesion scores moderately, with most animals surviving and improving substantially by day 14. With the same concentration of phosphate esters, however, there was a dramatic improvement in lesion scores, with all lesions resolved after 9 days, and all animals surviving, as shown in Figure 4.

EXAMPLE 13

15 Antiviral Effect Of Acyclovir Monophosphate In Mice Infected With An Acyclovir Resistant Wild Type HSV -1

The procedure of Example 12 was repeated using an acyclovir-sensitive, wild type HSV-1 and a formulation having only acyclovir monophosphate (ACV-MP) as the acyclovir derivative. Two creams were formulated, one having ACV-MP present in the aqueous cream at 14.5 millimoles/100 mL and the other having acyclovir present at 22.2 millimoles/100 mL (both 5 gm%, however, because of the addition of the phosphate group the number of moles of acyclovir present in the monophosphate is reduced relative to neat acyclovir).

20 Treatment was initiated 24 hours after infection and continued 4 times daily for four days. The ten untreated mice developed stage 4 lesions by the 5th day and all died by day 14 (Figure 5). The acyclovir monophosphate-treated animals did not develop lesions and 10 of 10 animals survived (Figure 5). In the acyclovir-treated group several animals developed mild lesions on days 7-9 which resolved; 9 of 10 ten animals survived.

25 This study shows that ACV-MP at a lower dosage (14.5 mmol/100 mL) was more effective than acyclovir (22.2 mmol/100 mL) in preventing lesions in wild type HSV-1 infection.

EXAMPLE 14

30 Antiviral Effect Of Acyclovir Monophosphate In Mice Infected With An Acyclovir Resistant HSV-1

The procedure of Example 12 was repeated using a formulation having only acyclovir monophosphate (ACV-MP) as the acyclovir derivative. Treatment was begun 3 hours post-infection, with treatments occurring twice on the day of infection, and thereafter, three times a day through day 4. Referring now to Figure 6, ACV-MP at 14.5 mmol/100 mL is clearly more effective than acyclovir at 22.2 mmol/100 mL in reducing lesion scores in animals infected with acyclovir-resistant (TK altered) HSV-1.

35 In the control and acyclovir-treated groups, 8 of 10 mice survived the 14 day experiment versus 10 of 10 surviving with acyclovir monophosphate treatment.

EXAMPLE 15

Antiviral Effect Of Acyclovir Monophosphate In
Guinea Pigs Infected With An Acyclovir Resistant HSV-2

5. We tested acyclovir monophosphate (ACV-MP) in aqueous cream (AC) to determine if it was more effective than 5% Acyclovir in polyethylene glycol (ACV-PEG) for treatment of a primary genital herpes. In particular, we studied a genital herpes infection of guinea pigs caused by an ACV-resistant HSV-2. Additionally, we compared acyclovir treatments in two carrier systems: aqueous cream (AC) and polyethylene glycol (PEG). The experiments were placebo-controlled and uninfected animals were treated with each ACV preparation to assess skin and vaginal irritation.

10. Intravaginal inoculation of weanling guinea pigs with HSV-2 results in a primary genital infection characterized by initial replication of virus in the vaginal tract followed by the development of external vesicular lesions. Virus titers peak on days one to three in the vaginal tract and gradually clear by days 7-10. The external genital lesions first appear on day four, peak lesion severity occurs on days 6-8, and the lesions generally heal by days 15-18.

15. Animals were inoculated with the HSV-2 strain 12247, which has an altered thymidine kinase and is resistant to *in vitro* treatment with ACV. Female Hartley guinea pigs (Charles River, Kingston, NY) weighing 250-300 grams were first vaginally swabbed to remove vaginal secretions. After one hour, the animals were inoculated intravaginally with 2.4×10^4 plaque forming units (pfu). Inoculation was accomplished by inserting a swab soaked with virus into the vaginal tract and rotating approximately six times.

20. Groups of 10 guinea pigs were treated both intravaginally and on the external genital skin with 0.1 mL (total of 0.2 mL per animal per treatment) of each preparation. Animals were treated three times daily for seven days beginning 24 hours post-viral inoculation. Three uninfected animals were treated with each preparation on the same schedule to assess local toxicity and irritation.

25. To determine the efficacy of the various treatments on HSV-2 replication in the vaginal tract, swabs of vaginal secretions were obtained during the primary infection on days 1, 3, 5, 7, and 10 after HSV-2 inoculation. The swabs were placed in tubes containing 2.0 mL of media, vortexed, and frozen at -70°C until titrated for HSV. When all samples were collected, they were thawed, diluted serially, and HSV-2 titers were determined using rabbit kidney cells in a microtiter CPE assay.

30. We also measured the development and severity of external genital lesions to determine the efficacy of treatment. Severity of lesions was graded on a 0-5+ score. The presence or absence and severity of lesions was recorded for 19 days after viral inoculation. Infection rates, peak lesion scores, peak virus titers, areas under virus titer-day curves, and lesion score-day curves between PBS placebo-treated and PEG drug-treated or AC placebo-treated and AC drug-treated animals were compared using the Mann-Whitney U rank sum test. A p-value of 0.05 or less was considered significant.

The effect of topical treatment with ACV preparations on vaginal viral replication is shown in Table 1. Only treatment with the ACV-MP preparations (5% ACV-MP-PEG or 5% ACV-MP-AC) significantly reduced

the virus titer-day area under the curve (AUC) and mean peak virus titers.

5

TABLE I

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**EFFECT OF TREATMENT WITH ACYCLOVIR MONOPHOSPHATE ON
VAGINAL VIRUS TITERS OF GUINEA PIGS INOCULATED
INTRAVAGINALLY WITH AN ACYCLOVIR RESISTANT HSV-2**

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Treatment ^A	# Virus Positive/# Inoculated	Virus Titer-Day Area Under Curve	P-Value	Mean Peak Virus Titer	P-Value
Placebo-PBS	10/10	31.6	—	5.0	—
Placebo-AC	10/10	34.6	NS ^B	5.1	NS
ACV-PEG	10/10	33.4	NS	5.2	NS
ACV-AC	10/10	27.9	NS	4.6	NS
ACV-MP-PEG	6/10	3.4	<0.001	2.0	<0.001
ACV-MP-AC	9/10	14.5	0.001	3.7	<0.05

25

A. Topical and intravaginal treatment was initiated 24 hours after viral inoculation and was continued three times daily for 7 days. Acyclovir content on a molar basis was lower in the tests conducted with acyclovir monophosphate (14.5 mmol/100 mL) versus those conducted with the neat acyclovir (22.2 mmol/100 mL).

30

B. NS - Not Statistically Significant when compared to the appropriate placebo-treated group.

35

The effect of topical treatment with ACV preparations on lesion development is depicted in Table II. Both ACV and ACV-MP preparations significantly altered the lesion score-day AUC when compared to the appropriate placebo-treated group. However, only therapy with 5% ACV-MP-PEG significantly reduced mean peak lesion scores.

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TABLE II

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**EFFECT OF TREATMENT WITH ACYCLOVIR MONOPHOSPHATE ON
EXTERNAL LESION DEVELOPMENT IN AN ACYCLOVIR RESISTANT
GENITAL HSV-2 INFECTION OF GUINEA PIGS**

Treatment A	Lesion Score-Day Area Under Curve	P-Value	Mean Peak Lesion Score	P-Value
Placebo-PBS	28.3	--	3.0	--
Placebo-AC	34.2	NS B	3.5	NS
ACV-PEG	9.5	0.001	1.8	NS
ACV-AC	19.3	0.01	2.5	NS
ACV-MP-PEG	1.7	<0.001	0.7	<0.001
ACV-MP-AC	23.4	<0.05	2.3	NS

10

A. Topical and intravaginal treatment was initiated 24 hours after viral inoculation and was continued three times daily for 7 days. Acyclovir content on a molar basis was lower in the tests conducted with acyclovir monophosphate (14.5 mmol/100 mL) versus those conducted with the neat acyclovir (22.2 mmol/100 mL).

20

B. NS - Not Statistically Significant when compared to the appropriate placebo-treated group.

25

In the guinea pig model of an ACV-resistant HSV-2 genital herpes infection, only ACV-MP significantly reduced vaginal viral replication. Also, the ACV-MP-PEG treated group had the lowest virus titer-day and mean peak titer values. While both ACV-MP and ACV altered lesion development, the drugs in PEG had lower scores than those in AC. Additionally, animals receiving ACV-MP-PEG had the lowest lesion score-day and mean peak lesion scores.

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Moreover, throughout the study, there were no signs of any irritation of the genital area or any other toxicity in uninfected ACV preparation-treated animals.

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These results demonstrate the strong activity of acyclovir monophosphate in treating HSV-2 genital herpes. Further, it is interesting to note that polyethylene glycol dispersed acyclovir monophosphate showed the best efficacy in treating lesions.

40

EXAMPLE 16

Activity Of Acyclovir Diphosphate

The procedure of Example 12 is repeated using a formulation having only acyclovir diphosphate (ACV-DP) as the acyclovir derivative. Efficacy superior to that of ACV alone is observed.

EXAMPLE 17**Activity Of Acyclovir Monophosphate Glycerol**

The procedure of Example 12 is repeated using a formulation having only acyclovir monophosphate glycerol (ACV-MP-G) as the acyclovir derivative. Efficacy superior to that of ACV alone is observed.

5

EXAMPLE 18**Activity Of Acyclovir Diphosphate Glycerol**

The procedure of Example 12 is repeated using a formulation having only acyclovir diphosphate glycerol (ACV-DP-glycerol) as the acyclovir derivative. Efficacy superior to that of ACV alone is observed.

10

EXAMPLE 19**Activity Of Acyclovir Monophosphate Morpholidate**

The procedure of Example 12 is repeated using a formulation having only acyclovir monophosphate morpholidate (ACV-MP-morpholidate) as the acyclovir derivative. Efficacy superior to that of ACV alone is observed.

EXAMPLE 20

15

Activity Of Acyclovir Monophosphate Isopropylidene Glycerol

The procedure of Example 12 is repeated using a formulation having only acyclovir monophosphate isopropylidene glycerol (ACV-MP-isoP-G) as the acyclovir derivative. Efficacy superior to that of ACV alone is observed.

20

EXAMPLE 21**Activity Of Acyclovir Diphosphate Isopropylidene Glycerol**

The procedure of Example 12 is repeated using a formulation having only acyclovir diphosphate isopropylidene glycerol (ACV-DP-isoP-G) as the acyclovir derivative. Efficacy superior to that of ACV alone is observed.

25

EXAMPLE 22**Activity Of Acyclovir-Phosphomethylenediphosphonate and Acyclovirtriphosphate**

The procedure of Example 12 is repeated using a formulation having only ACV-Phosphomethylenediphosphonate and Acyclovirtriphosphate, independently, as the acyclovir derivative. Efficacy superior to that of ACV alone is observed.

EXAMPLE 23

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Solubility Of Acyclovir Monophosphate And Salts

Various salts of acyclovir monophosphate were tested for solubility as follows:

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Two mL of distilled water was placed in each of three 10 mL beakers, each beaker having magnetic stirring bars. In each individual flask, an acyclovir monophosphate salt, selected from potassium, sodium, sodium/ammonium, and H⁺ (free acid), was added until a saturated solution was formed. Each saturated salt solution was gravity filtered. The acyclovir monophosphate sodium/ammonium and free acid salt solutions were filtered through Whatman No. 4 filter paper and gave clear solutions. The potassium and

sodium salt solutions were filtered through Whatman No. 1 filter paper and each gave slightly opalescent solutions.

One mL of each of the saturated salt solutions were transferred by pipette to preweighed round bottom flasks, and the solutions were allowed to dry. After all of the liquid had evaporated, the round bottom flasks were reweighed and the number of milligrams of acyclovir monophosphate salt present per milliliter was easily found.

5 The following Table sets forth the solubility of the various salts prepared as described above relative to acyclovir:

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TABLE III

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Salt	Solubility as Compared to Acyclovir
H ⁺	21 X
K ⁺	85 X
Na ⁺ /NH ₄ ⁺	100 X
Na ⁺	108 X

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It will be appreciated in view of the results shown in Table III, that through formation of a salt of the acyclovir monophosphate, solubility can be dramatically enhanced. It is expected that other nucleoside monophosphates will exhibit similarly enhanced solubility. In this manner, it is possible to formulate topical compositions containing large quantities of acyclovir monophosphate because of the enhanced solubility of the salts.

WHAT IS CLAIMED IS:

1. An antiviral nucleoside analogue phosphate ester selected from the group consisting of antiviral nucleoside analogue phosphorothioates, nucleoside analogue phosphoamidates, and nucleoside analogue phosphofluoridates.
- 5 2. An antiviral nucleoside analogue phosphate ester selected from the group consisting of 1,2- μ -methylene-nucleoside analogue diphosphates; 2,3- μ -methylene-nucleoside analogue triphosphates; 1,2- μ -thio-nucleoside analogue diphosphates; and 2,3- μ -thio-nucleoside analogue triphosphates.
- 10 3. A compound according to Claim 1 or 2 wherein the antiviral nucleoside analogue is acyclovir, 9-(2-hydroxyethoxymethyl)-guanine.
4. A pharmaceutical composition comprising an effective antiviral amount of an antiherpes nucleoside analogue phosphate ester according to any of Claims 1-3 or pharmaceutically acceptable salt thereof, or mixtures thereof, in a pharmaceutically acceptable carrier for topical use.
- 15 5. The composition of Claim 4 wherein said nucleoside analogue phosphate ester is selected from the group consisting of nucleoside analogue phosphorothioates, nucleoside analogue phosphoamidates, and nucleoside analogue phosphofluoridates.
6. The composition of Claim 4 wherein said nucleoside analogue phosphate ester is selected from the group consisting of 1,2- μ -methylene-nucleoside analogue diphosphates; 2,3- μ -methylene-nucleoside analogue triphosphates; 1,2- μ -thio-nucleoside analogue diphosphates; and 2,3- μ -thio-nucleoside analogue triphosphates.
- 20 7. The composition of Claims 4-6 wherein said nucleoside analogue is acyclovir.
8. The composition of any of Claims 4-6, wherein said nucleoside analogue phosphate ester is acyclovir monophosphate.
- 25 9. The composition of any of Claims 4-6 wherein said nucleoside analogue phosphate ester is acyclovir diphosphate.
10. The composition of any of Claims 4-6, wherein said nucleoside analogue phosphate ester is acyclovir triphosphate.
- 30 11. The formulation of any of Claims 4-6, wherein said anti-herpes nucleoside analogue is selected from the group consisting of 1-beta-D-arabinofuranosyl-5-(2-bromovinyl)uracil; 2'-fluorocarbocyclic-2'-deoxyguanosine; 6'-fluorocarbocyclic-2'-deoxyguanosine; 1-(beta-D-arabinofuranosyl)-5(E)-(2-iodovinyl)uracil;

(1*r*-1*α*, 2*β*, 3*α*)-2-amino-9-(2,3-bis(hydroxymethyl)cyclobutyl)-6*H*-purin-6-one;
9*H*-purin-2-amine, 9-((2-(1-methylethoxy)-1-((1-methylethoxy)methyl)ethoxy)methyl)-(9CI);
trifluorothymidine;
9-((1,3-dihydroxy-2-propoxy)methyl)guanine;
5-ethyl-2'-deoxyuridine;
E-5-(2-bromovinyl)-2'-deoxyuridine;
5-(2-chloroethyl)-2'-deoxyuridine;
1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-iodocytosine (FIAC);
1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-iodouridine (FIAU);
buciclovir;
6-deoxyacyclovir;
9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine;
E-5-(2-iodovinyl)-2'-deoxyuridine;
5-vinyl-1-beta-D-arabinofuranosyluracil;
1-beta-D-arabinofuranosylthymine;
2'-nor-2'deoxyguanosine;
9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine;
1-beta-D-arabinofuranosyladenine.

12. A composition according to any one of Claims 4-6, wherein the nucleoside analogue phosphate ester is in the form of a pharmaceutically acceptable salt.

20 13. A composition according to Claim 11, wherein the salt is selected from the group consisting of sodium, potassium, ammonium, and hydrogen salts.

14. A composition according to any of Claims 4-6, wherein the pharmaceutical carrier is selected from the group consisting of an aqueous cream and polyethylene glycol.

25 15. A method for the topical treatment of the cutaneous or mucosal lesions of a herpes virus infected animal, comprising applying an effective amount of a composition containing an effective amount of an antiviral nucleoside analogue according to any of Claims 4-6 to the affected lesions.

16. A method according to Claim 15, wherein the nucleoside analogue phosphate ester is acyclovir monophosphate.

30 17. A method according to Claim 15, wherein said nucleoside analogue phosphate ester is acyclovir diphosphate.

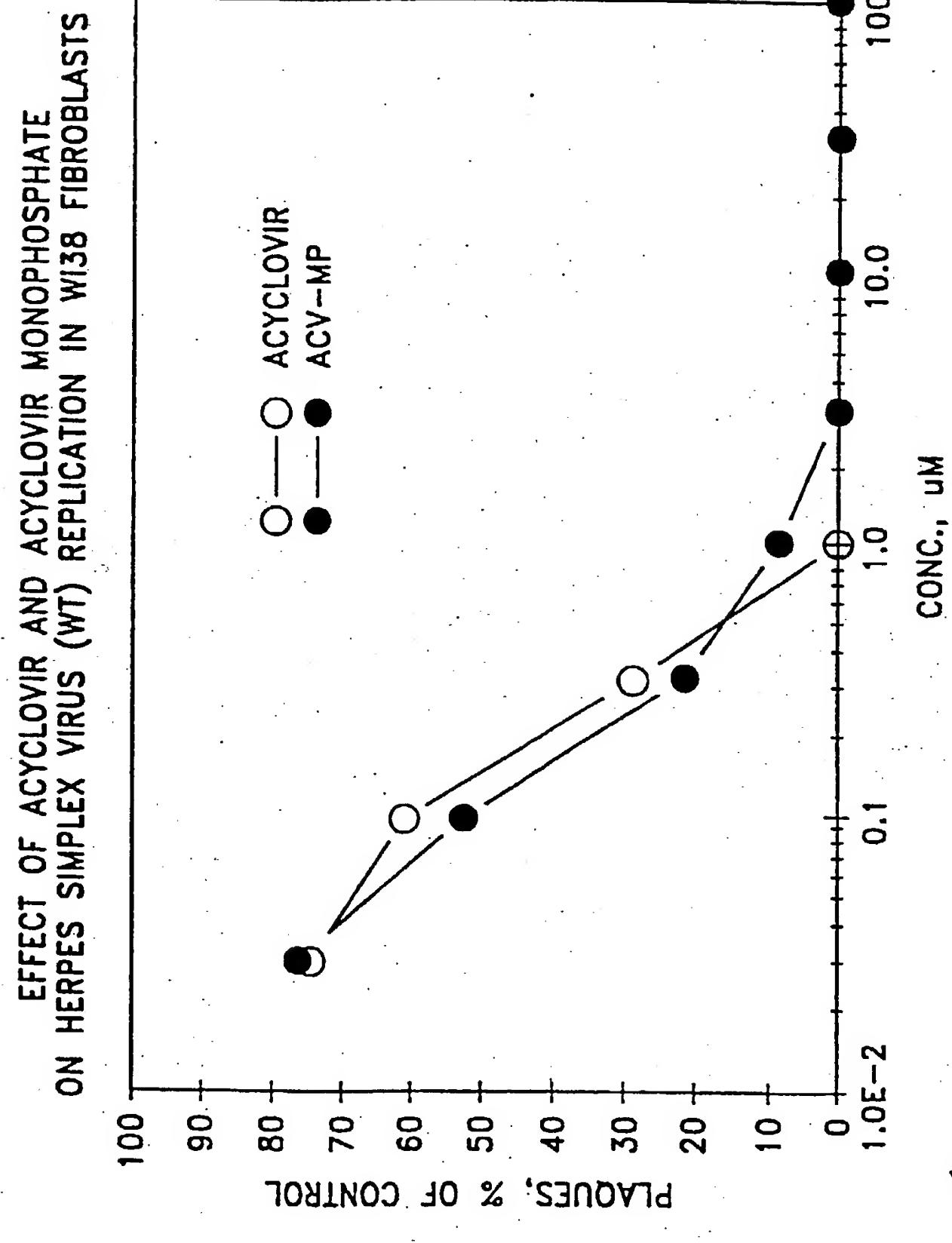
18. A method according to Claim 15, wherein said nucleoside analogue phosphate ester is acyclovir triphosphate.

35 19. A method for treating a herpes virus infection in which the herpes virus has developed a resistance to one or more antiviral compounds due to an alteration or defect in the viral gene coding for thymidine kinase, comprising applying an effective amount of an antiherpes virus nucleoside analogue

phosphate ester, a pharmaceutically acceptable antiherpes virus nucleoside analogue phosphate ester salt, or a mixture thereof, in a pharmaceutical carrier suitable for topical use, to the herpes virus-infected cutaneous or mucosal tissues of an animal.

20. A method according to Claim 15 wherein said animal is a human being.
- 5 21. A method according to Claim 15, wherein said infection is due to a strain of herpes virus that is acyclovir-resistant.
22. A method according to Claim 15, wherein said acyclovir-resistance of said virus is due to an alteration or defect in the viral gene coding for thymidine kinase.
- 10 23. A method for the treatment of a herpes virus infection in an animal, comprising topically applying a composition containing an effective antiviral amount of acyclovir triphosphate to the affected cutaneous or mucosal lesions of the animal.

1/6



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Fig. 1

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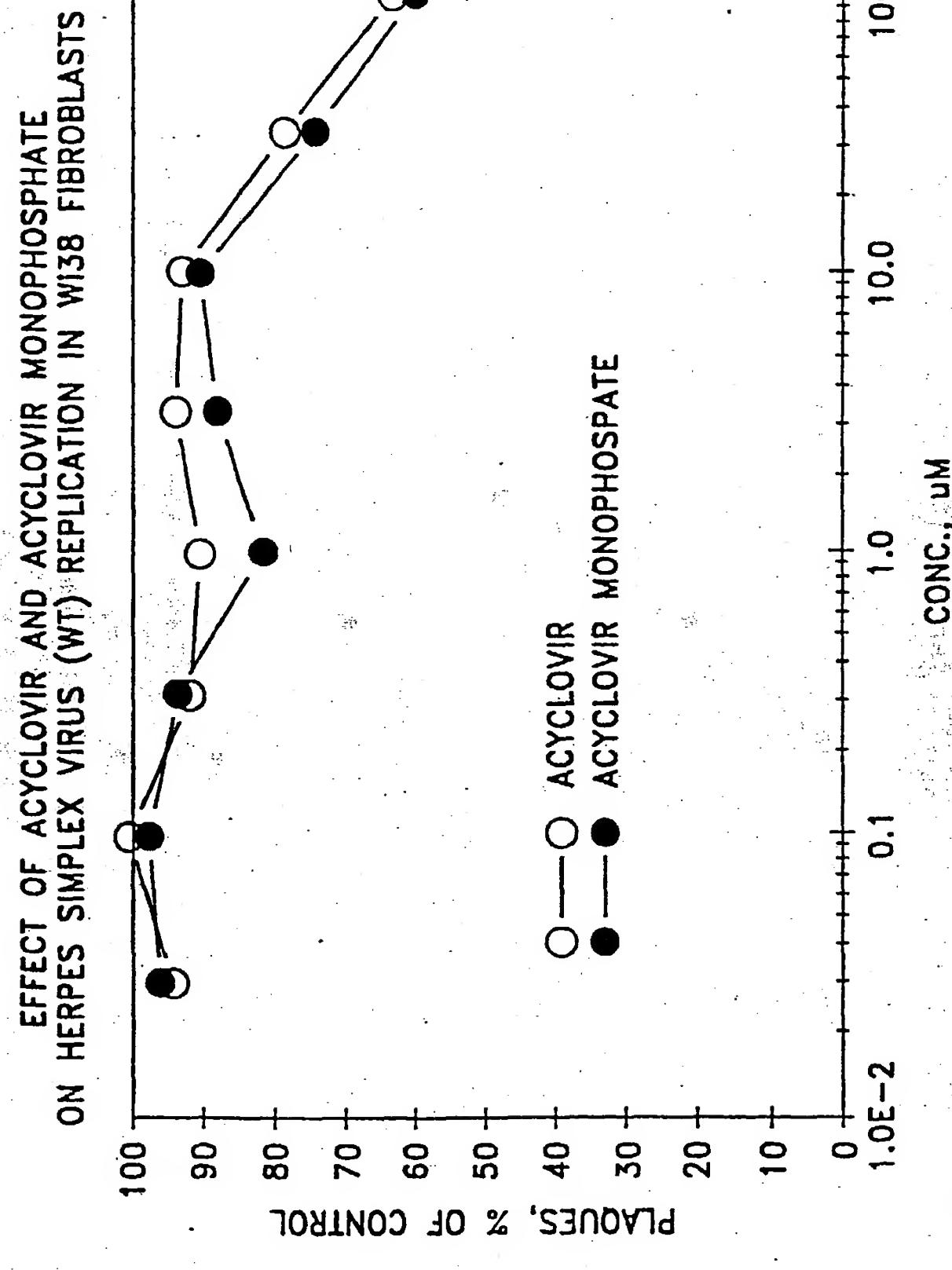
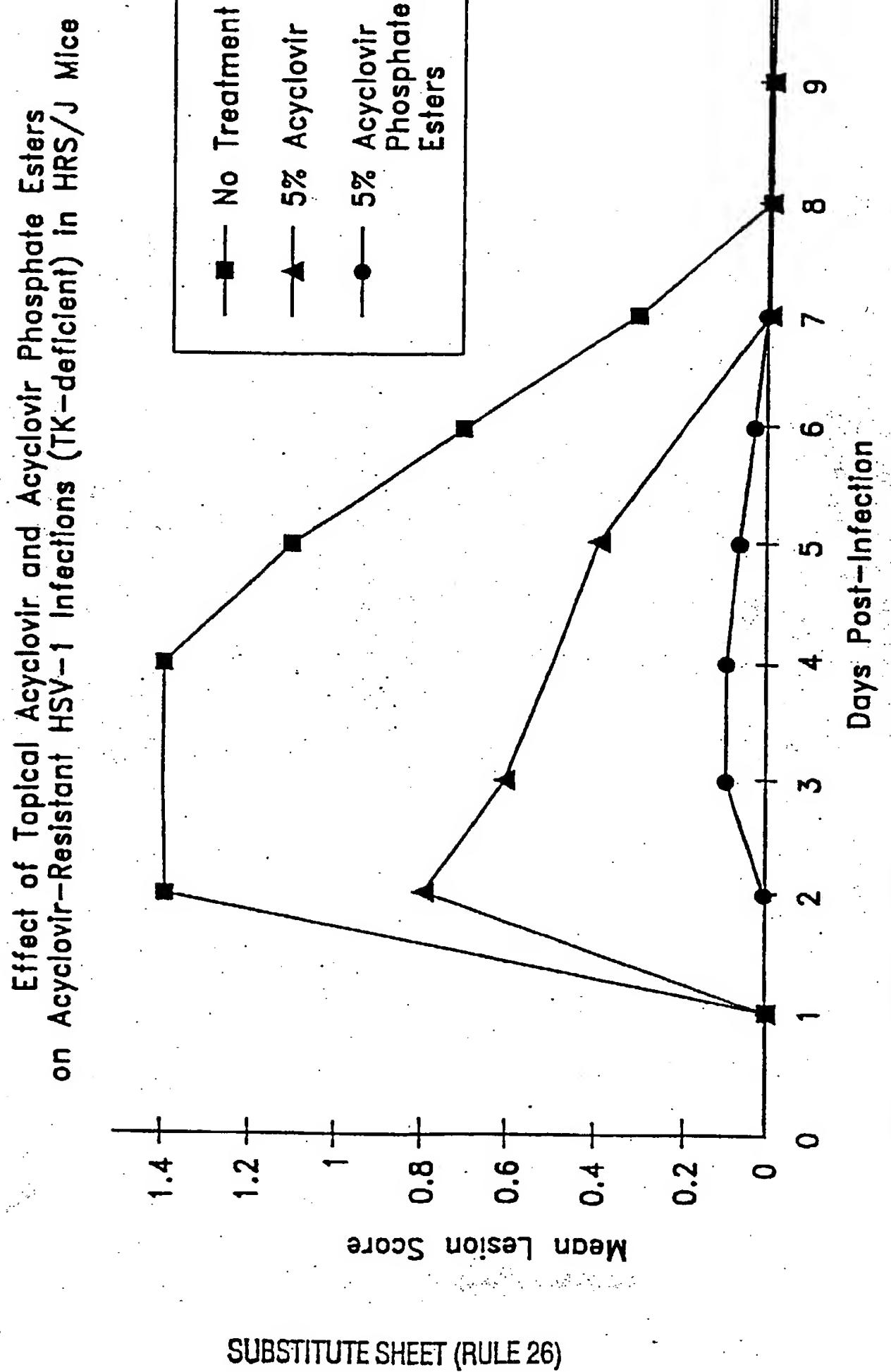


Fig. 2

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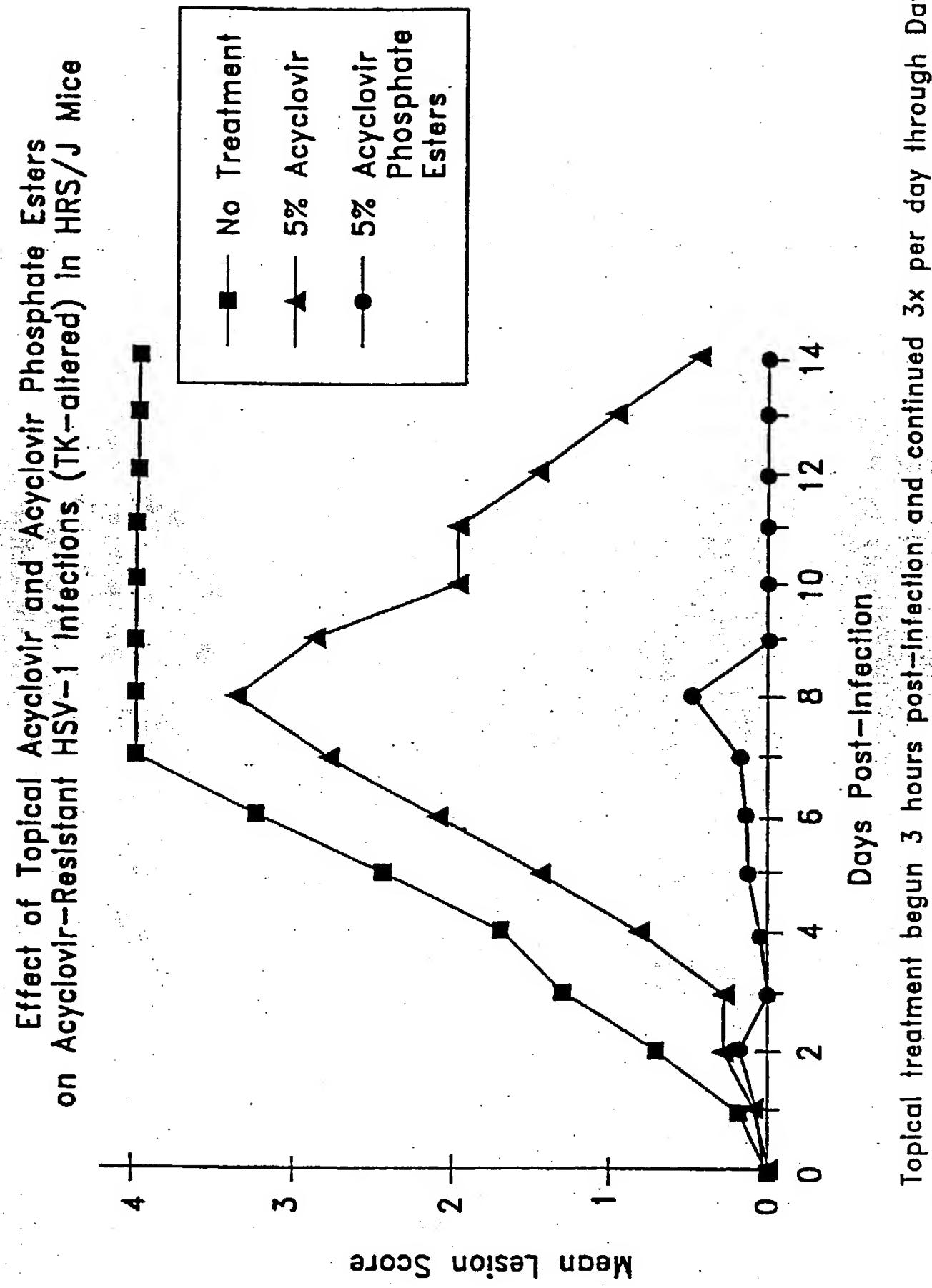
3/6



Topical treatment begun 3 hours post-infection and continued 3x per day through Day 4

Fig. 3

4/6



Topical treatment begun 3 hours post-infection and continued 3x per day through Day 5

Fig. 4

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EFFECT OF TOPICAL ACYCLOVIR AND
ACYCLOVIR MONOPHOSPHATE ON HERPES SIMPLEX
INFECTION (HSV-1, wild type) IN HRS/J MICE

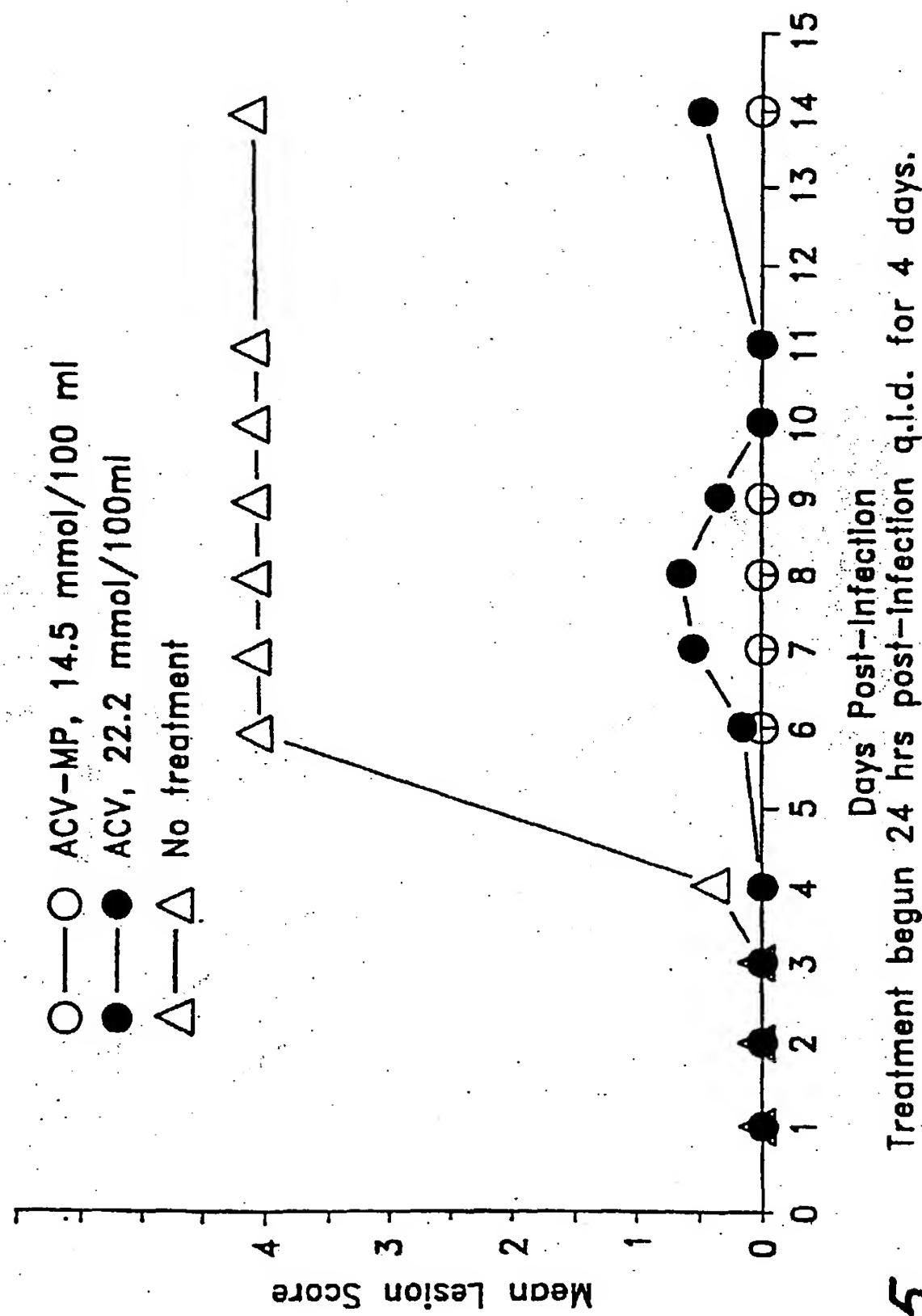
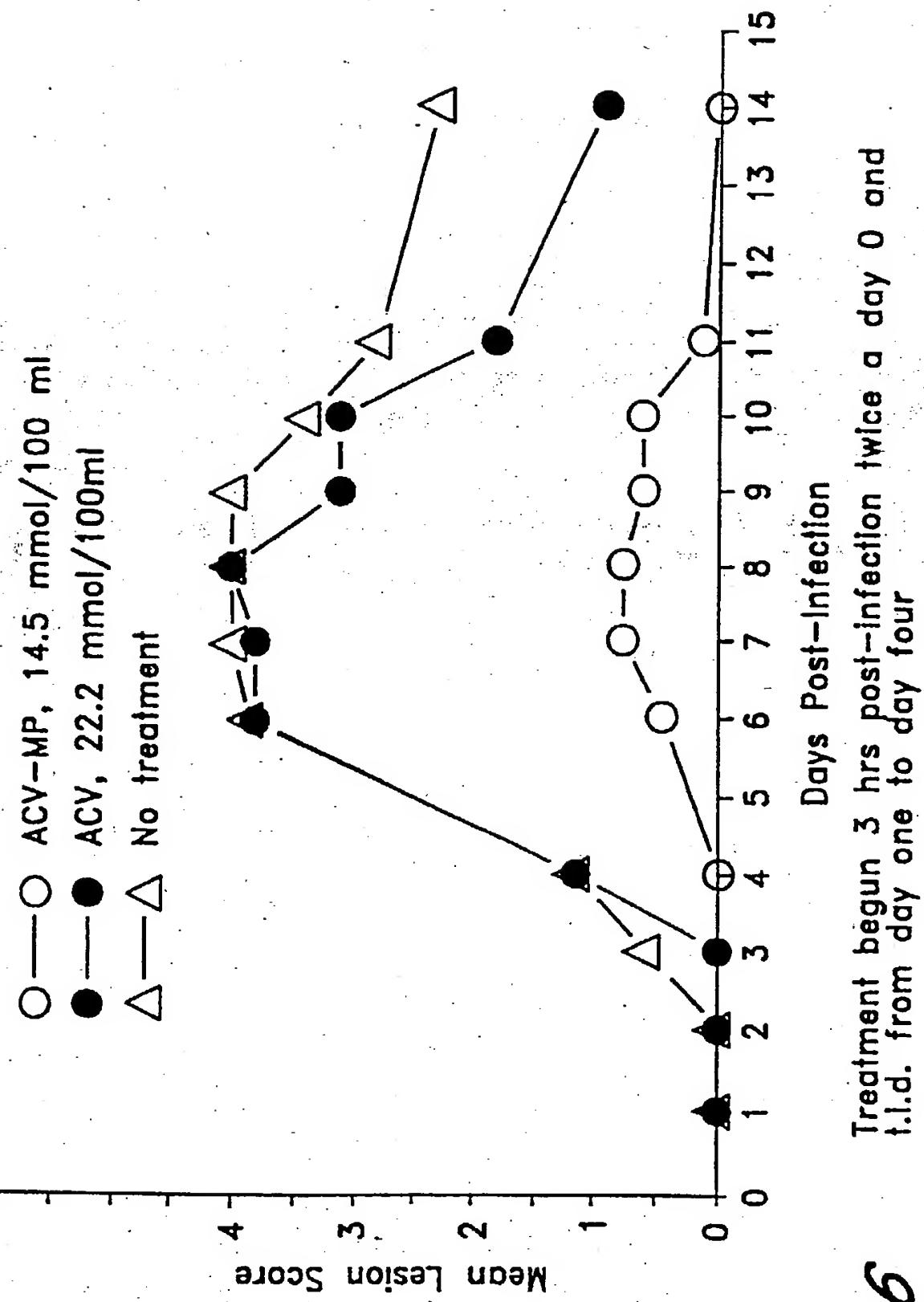


Fig. 5

Treatment begun 24 hrs post-infection q.i.d. for 4 days.

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EFFECT OF TOPICAL ACYCLOVIR AND
ACYCLOVIR MONOPHOSPHATE ON ACYCLOVIR-RESISTANT
HSV-1 INFECTION (TK-altered) IN HRS/J MICE



Treatment begun 3 hrs post-infection twice a day 0 and t.i.d. from day one to day four

Fig. 6

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10085

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 9/70

US CL : 424/443

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/443

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

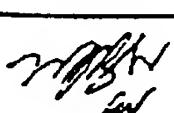
C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,146,715 A (SCHAEFFER) 27 March 1979, column 1, lines 12-13, 21-35; column 3, lines 39-43.	1-18
—		—
Y		1-23
X	US 4,199,574 A (SCHAEFFER) 22 April 1980, column 1, lines 12-13, 21-35; column 3, lines 39-43.	1-18
—		—
Y		1-23

Further documents are listed in the continuation of Box C. See patent family annex.

Special categories of cited documents:	T	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance		
"E" earlier document published on or after the international filing date	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
07 AUGUST 1996	26 SEP 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Carlos Azpuru  Telephone No. (703) 308-2351
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